

CLINICAL SCIENCE

The role of oxidative stress and antioxidants in the pathogenesis of age-related macular degeneration

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OBJECTIVE: To investigate the role of oxidant/antioxidant status and protein oxidation in the development of age-related macular degeneration.

METHOD: The activities of serum superoxide dismutase and glutathione peroxidase and the levels of serum malondialdehyde, advanced oxidation protein products, glutathione and vitamin C were measured in 25 patients with age-related macular degeneration and 25 control subjects without age-related macular degeneration.

RESULT: The malondialdehyde and advanced oxidation protein product levels in the serum were significantly higher in the age-related macular degeneration patient group than in the control group ($p < 0.05$). The superoxide dismutase activity in the serum was significantly lower in the age-related macular degeneration patient group than in the control group ($p < 0.05$). The levels of vitamin C and glutathione and the activity of glutathione peroxidase in the serum were unchanged between groups ($p > 0.05$).

CONCLUSION: The results of the present study suggest that decreased effectiveness of the antioxidant defense system and increased oxidative stress may play a role in the pathogenesis of age-related macular degeneration.

KEYWORDS: Oxidative stress; Antioxidants; Protein oxidation; AMD.

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment and blindness among people aged 60 years and older.¹ Although the disease presents a serious social and economic problem, its pathogenesis and etiology are still unclear.^{2,3} Based on the disease's clinical and pathological features, there are two subtypes of late AMD: atrophic (dry) and neovascular (wet), both of which can lead to significant visual loss.⁴

Over the past decade, the body of literature regarding the modifiable factors associated with AMD has grown considerably and includes cigarette smoking, age,⁵ nutritional factors,⁶⁻⁸ obesity⁹ and insufficient antioxidants in the diet.¹⁰

The eye is an exceptional organ because of its continuous exposure to environmental chemicals, radiation, and atmospheric oxygen.¹¹ These oxidative stresses have been implicated in the possible pathophysiology of various ocular diseases, such as AMD, cataracts, glaucoma, uveitis, and pseudoexfoliation syndrome. Reactive oxygen species

(ROS) are involved in this process. Several ocular degenerative disorders have been studied, and the presence of oxidative stress has been demonstrated through markers of lipid peroxidation, the activity of antioxidant enzymes, and the levels of low-molecular-weight antioxidants.¹²

Non-enzymatic lipid peroxidation is an example of a free radical-associated process through which oxidative stress promotes cellular damage. Serum malondialdehyde (MDA) is the end product of the primary reactions that lead to the significant oxidation of such polyunsaturated fatty acids in cellular membranes and, thus, serves as a reliable marker of oxidative stress.¹³ Protein oxidation is currently considered to be an important factor in a variety of diseases, such as Alzheimer's and Parkinson's diseases, cancer, hypertension, cardiovascular disease, diabetes, ischemia-reperfusion injury and aging.^{14,15}

Advanced oxidation protein products (AOPP) are described as dityrosines that contain cross-linked protein products. Importantly, this definition excludes protein aggregates that form as a result of disulfide links following low-level oxidative stress. Therefore, the presence of AOPP may be a better and more accurate marker of oxidative stress than lipid peroxidation products.¹⁶

Endogenous antioxidants, including such non-enzymatic scavengers as glutathione (GSH) and such antioxidant enzymes as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), are the first lines of

defense against oxidative stress and act by scavenging potentially damaging free radical moieties.¹⁷

Ascorbate (vitamin C) is the most effective aqueous-phase antioxidant found in human blood. Increasing evidence suggests that, within the aqueous phase, vitamin C plays a vital role in the antioxidant defense mechanism of the eye, protecting ocular tissues against photooxidative damage by acting as a free radical scavenger.¹⁸

Determining the level of oxidative stress encountered by the human eye has not yet been attempted in clinical diagnoses and/or treatment.¹⁹ The aim of the present study was to investigate the role of antioxidants and protein and lipid peroxidation in the development of AMD. Therefore, we measured the activities of the SOD and GPx enzymes and the serum levels of MDA, AOPP, GSH and vitamin C in patients with AMD and in control subjects not exhibiting AMD.

MATERIALS AND METHODS

This study included 15 men and 10 women (mean age \pm SD; 65.16 ± 13.96 years) with AMD and choroidal neovascular (CNV) membrane, which is secondary to AMD. The control group included 15 men and 10 women (mean age \pm SD; 65.72 ± 12.62 years). No statistically significant differences between the groups were observed in terms of age and sex. Patients with other ophthalmic conditions (e.g., glaucoma, uveitis, pseudoexfoliation syndrome, other progressive retinal diseases) and systemic diseases (e.g., diabetes, arthritis, coronary arterial disease, peripheral vascular disease) were excluded.

All subjects, both in the control group and the patient group, completed a questionnaire confirming the following information: age, gender, non-smoker status, non-consumption of supplements, such as vitamins and/or antioxidants. All patients with AMD were first tested to ensure that they did not ingest antioxidant vitamins and minerals. After obtaining blood samples from the patients, we initiated anti-vascular endothelial growth factor therapy.

All patients underwent a comprehensive ophthalmic examination. CNV was diagnosed by slit-lamp biomicroscopy of the fundi, color fundus photographs, fundus fluorescein angiographies, and optical coherence tomographies. The control subjects exhibited entirely normal ophthalmic conditions. For this study, the rules of the Human Ethics Committee were followed. Blood samples, which were drawn from patients and control subjects after an overnight fast and before the operation, were centrifuged at $2,000 \times g$ for 10 min at 4°C . Serum samples were stored at -70°C for further analysis.

AOPP levels were measured by a spectrophotometric method (Shimadzu UV 1601 spectrophotometer, Shimadzu, Tokyo, Japan) in the presence of potassium iodide at 340 nm ¹⁶ and using chloramine-T solutions for calibration. AOPP levels were expressed in micromoles of chloramine-T equivalents per liter.

Lipid peroxidation was estimated using the thiobarbituric acid-reactive substances (TBARS) test, as described previously.²⁰ Briefly, the formation of TBARS was quantitated using 1,1,3,3-tetraethoxypropane as a standard, and the absorbances of the TBARS were read at 532 nm using a Shimadzu UV 1601 spectrophotometer (Tokyo, Japan).

The GSH levels were determined as total sulfhydryl group (R-SH).²¹ A 0.5 ml aliquot of each sample was mixed with 1 ml of a solution containing 100 mM Tris-HCl pH 8.2, 1% sodium dodecyl sulfate (SDS) and 2 mM EDTA. The

mixture was incubated for 5 min at 25°C and centrifuged to remove any precipitate. Next, 5,5-dithiobis (2-nitrobenzoic acid)/DTNB 0.3 mM was added to each reaction volume, and the reaction was incubated for 15 min at 37°C . The absorbance of each sample was determined at 412 nm . The R-SH levels were calculated assuming a molar extinction coefficient of $13,000\text{ mol}^{-1}\text{ cm}^{-1}$ at 412 nm .

The SOD activity was measured by inhibiting this activity through nitroblue tetrazolium (NBT) reduction. Xanthine-xanthine oxidase was used as a superoxide generator, and one IU was defined as the quantity of SOD required to produce a 50% inhibition.²²

The level of GPx activity was determined spectrophotometrically as described in the literature.²³ The reaction mixture, containing a 50 mM phosphate buffer pH 7.4, 7.7 units of GSH reductase, 5 mM GSH, and crude extract, was preincubated for 5 min at 37°C . Next, 20 μl of a nicotinamide adenine dinucleotide phosphate solution in the reduced form (NADPH) (0.3 mM) was added, and the hydroperoxide-independent consumption of NADPH was monitored for approximately 5 min. The overall reaction was started by adding 20 μl of prewarmed hydroperoxide solution (0.025 mM), and the reduction in absorption at 340 nm was monitored.

The level of total ascorbate was determined by the modified Roe and Kuether method.²⁴ Serum samples were added to a trichloroacetic acid solution and centrifuged at $3,000 \times g$ for 10 min. Next, a 2,4-dinitrophenylhydrazine (DNPH)-thiourea-copper sulfate reagent was added to the serum sample tubes. The contents of each tube were mixed, capped with parafilm, and placed in a water bath at 37°C for 4 h, after which the tubes were removed and cooled in ice water. An ice-cold 65% sulfuric acid solution was added to each tube, and the sample was mixed thoroughly. The mixture was allowed to stand at room temperature for 30 min, and the absorbance of each tube was read in a Shimadzu UV 1601 model spectrophotometer at 515 and 520 nm. The lower limit of detection for vitamin C was $0.05\text{ }\mu\text{mol/L}$.

Statistical Analysis. Data are presented as the mean \pm SD. Statistical analyses were conducted by Kruskal Wallis test and Mann-Whitney *U*-test (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). A value of $p < 0.05$ was defined as significant.

RESULTS

This study included a total of 50 subjects who were equally divided among two groups, an AMD group and a control group. The mean age was 65.16 ± 13.96 years (40-89 years) (for 15 men and 10 women) in the AMD group and 65.72 ± 12.62 years (36-89 years) (for 15 men and 10 women) in the control group. All of the groups were matched for age and gender, and no statistically significant differences were observed. The levels of MDA and AOPP in the serum were significantly higher in the AMD patient group than in the control group ($p < 0.05$) (Table 1). Furthermore, the level of SOD activity in the serum was significantly lower in the AMD patient group than in the control group ($p < 0.05$) (Table 1). In addition, the levels of vitamin C and GSH and the activities of GPx in the serum were unchanged between the two groups ($p > 0.05$) (Table 1).

DISCUSSION

AMD is a complex, multifactorial disease of aging for which several theories of pathogenesis have been proposed,

Table 1 - Oxidation markers are down-regulated in AMD patients and Control Subjects (mean \pm SD).

	AMD group (n = 25)	Control group (n = 25)
SOD (U/mL)	4.99 \pm 3.09*	9.91 \pm 4.42
GPx (U/mL)	12.97 \pm 11.23	15.79 \pm 11.20
MDA (nmole/L)	6.39 \pm 1.80*	4.90 \pm 1.97
AOPP (μ mole/L)	251.89 \pm 69.58*	181.43 \pm 43.11
GSH (nmole/mL)	310.05 \pm 102.51	331.03 \pm 129.45
Vitamin C (μ mole/L)	2.54 \pm 1.50	3.14 \pm 1.54

* $p < 0.05$, as compared with the control group.

including oxidative damage²⁵ and ocular perfusion abnormalities.²⁶

Oxidative stress may cause injury to the retinal pigment epithelium (RPE), the Bruch's membrane, and the choroid, which are layers in the eye involved in the pathophysiology of AMD.²⁷⁻³¹

Liang et al.³² demonstrated that human RPE cells exposed to oxidative stress or rod outer segments exhibited damage primarily to mitochondrial (mt) DNA and that damaged mtDNA was not efficiently repaired.

The retina is particularly susceptible to oxidative stress due to its high concentration of oxygen, its high proportion of polyunsaturated fatty acids, and its exposure to visible light.³³ Prior reports have suggested that the retina is susceptible to lipid peroxidation^{34,35} and that this susceptibility also increases with aging in the macular region.³⁵

Previous studies demonstrated that the plasma MDA levels were higher in an AMD patient group than in a control group.^{36,37} In the present study, we found that the serum MDA levels were significantly higher in the AMD patient group than in the control group, in agreement with a previous study.³⁸

Protein oxidation is also a useful marker for the evaluation of oxidative stress in vivo. Many different types of protein oxidative modifications can be induced by free radicals. A prior study demonstrated that the levels of protein carbonyl groups in the serum were higher in the AMD patient group than in the control group.³⁸

AOPP measurements reflect the generation of free radicals and the degree of protein oxidation.^{16,39} In the present study, we found that the levels of AOPP in the serum were significantly higher in the AMD patient group than in the control group. Therefore, this study demonstrated that protein oxidation, a useful oxidative stress marker, is upregulated in AMD, suggesting increased oxidative stress.

Antioxidant enzymes are a primary defense system that protects biological macromolecules from oxidative damage. SOD, CAT and GPx are antioxidant enzymes that form part of the complex system that protects the retina from oxidative damage, and all three of these enzymes are found in the photoreceptors and the RPE.⁴⁰ SOD is a key antioxidant enzyme involved in the metabolism of oxygen free radicals.⁴¹ A previous report suggested that the activities of SOD in plasma and erythrocytes were lower in the AMD patient group than in the control group.³⁷ In the present study, we found that the activities of SOD in the serum were significantly lower in the AMD patient group than in the control group.

GSH is a major non-enzymatic antioxidant that is effective in protecting cells against to reactive oxygen products and toxins. A high concentration of GSH is present in most

living cells, and GSH is involved in the responses to various stresses. The redox condition of cells is associated with their degree of protection by GSH in the reduced state.⁴²

A prior study demonstrated that the plasma GSH levels were lower in an AMD patient group than in a control group.⁴³ In the present study, however, we determined that the serum GSH levels were unchanged between our groups.

GPx uses GSH as an electron donor to reduce organic hydroperoxides. GPx is found in the human retina and is dependent on selenium as a cofactor. Extracellular GPx is found in the retina, ciliary epithelium, and aqueous humor, and GPx may act as an extracellular antioxidant.³³

A previous study demonstrated that the activities of GPx in the plasma and erythrocytes were lower in an AMD patient group than in a control group.^{37,43} In the present study, the GPx activities in the serum were unchanged between groups.

Vitamin C is the second major water-soluble antioxidant. An important function of vitamin C is protecting the lens by means of direct absorption of ultraviolet radiation by aqueous vitamin C.⁴⁴

A prior study demonstrated that the low levels of vitamin C in the plasma were associated with an increased risk of AMD but that high levels were not protective.⁴⁴ In the present study, the serum vitamin C levels were unchanged between groups.

Much of the research into the relationship between oxidative stress and AMD has focused on the antioxidant status of subjects with and without the disease, and the limitations of these studies are worth noting.

Deficiencies in antioxidant enzymes may be related to the development of disease. Because the retina suffers oxidative damage, antioxidant nutrients are thought to be protective of the retina through their function as antioxidants.

The results of the present study suggest that reductions in the antioxidant defense system and increased oxidative stress may play a role in the pathogenesis of AMD.

In conclusion, increased oxidative stress, which causes oxidative damage to lipids and proteins and decreases antioxidant capacity, may lead to irreversible damage in the form of AMD. Further studies that analyze samples obtained both from the serum and the aqueous humor are required to confirm our findings.

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